REACTION OF COMPLEMENT WITH ENDOTHELIAL CELLS IN A MODEL OF XENOTRANSPLANTATION

A.P.Dalmasso[1,3], J.L.Platt[2,4,5] and F.H.Bach[2,3,6].

[1]VA Medical Center and [2]Immunology Research Center, [3]Department of Laboratory Medicine and Pathology, and Departments of [4]Pediatrics, [5]Cell biology and Neuroanatomy, and [6]Surgery, University of Minnesota Medical School, Minneapolis, MN55417, USA.

SUMMARY

We review our studies on the role of complement (C) as mediator of xenograft hyperacute rejection using an *in vitro* model consisting of porcine endothelial cells as target and human serum as source of natural antibodies and C. Cytotoxicity of endothelial cells required IgM antibodies to porcine endothelial cells, and the classical pathway and membrane attack complex of C. These findings correlated with *in vivo* results of porcine organs transplanted into rhesus monkeys, which showed a) co-deposition of IgM, C3, C4 and C9, along blood vessels of rejecting organs, with trace deposits of factors B or P, and b) minimal deposition of IgM and C components in transplants with prolonged survival that were performed in rhesus monkeys depleted of natural antibodies but with normal C levels. Human serum causes activation of porcine endothelial cells manifested by release of heparan sulfate proteoglycan. Heparan sulfate release was induced by C5a alone. A new approach to avert xenograft hyperacute rejection was tested. To inhibit cytotoxicity of porcine endothelial cells by human C, the membrane-associated C inhibitor decay-accelerating factor (DAF) of human origin was incorporated into endothelial cells. Human DAF was able to efficiently inhibit C-mediated killing of porcine endothelial cells, suggesting that the use of DAF and other C inhibitors could be used to interfere with C-mediated xenograft hyperacute rejection.

INTRODUCTION

The complement (C) system is considered to play a central role in the pathogenesis of hyperacute rejection of xenogeneic organs such as the heart or kidney (reviewed in 1,2). The participation of C in hyperacute rejection has been demonstrated with decomplemented or C-deficient animals, which showed prolongation in the survival of organ xenografts in comparison to controls with normal C levels (3-7). However, the mechanisms of C participation in xenograft hyperacute rejection are poorly understood. We carried out studies on the role of C in xenotransplantation in a model that potentially might be relevant for clinical application to humans. We used cultured porrcine endothelial cell monolayers to represent target cells of the hyperacute rejection reaction and human serum as source of natural antibody and C (2,8,9). Herein we present a summary of our studies, which in part have been published before. Our objectives were to investigate in the swine to primate model: A) the mechanism of C activation when porcine endothelial cells are incubated with human serum (10), B) the participation of antibody and C in endothelial cell activation manifested by release of heparan sulfate proteoglycan (8,11,12), and C) the feasibility of using a novel approach to inhibit rejection by incorporating host membrane-associated C inhibitors into xenogeneic endothelial cell membranes (13).

MECHANISM OF COMPLEMENT ACTIVATION

Immunofluorescence microscopy of tissues from rejecting porcine hearts and kidneys transplanted into untreated rhesus monkeys showed deposition of C3, C4, C5 and C9 along endothelial surfaces, with a distribution similar to that of IgM. Trace deposits of IgG and IgA, and factors B and P, appeared late in the course of rejection. Thus, hyperacute rejection of porcine organs by rhesus is accompanied by endothelial cell binding of IgM and C components of the classical pathway and membrane attack complex but minimal deposits of alternative pathway proteins (10,2,14).

In vitro studies were then carried out to investigate in detail the mechanism of C activation, using ^{51}Cr-labelled porcine endothelial cell monolayers as a target and human serum as source of natural antibody and C. Normal human serum was cytotoxic to porcine endothelial cells but cytotoxicity was abrogated by pre-incubation of the human serum at 56^{o}C for 30 min. Human serum immunochemically depleted of IgM retained normal C level but lost the ability to cause cytotoxicity, which was restored in a

dose-dependent manner by reconstitution with purified human IgM (10). Thus the experiments showed that natural antibodies trigger C-mediated lysis.

Participation of the classical pathway was demonstrated by the inability of C2-deficient serum to cause endothelial cell cytotoxicity, which was restored by reconstitution with purified C2. The alternative pathway does not appear to be primarily involved, as factor B-depleted serum caused cytotoxicity comparable to that induced by unmodified serum. Cytotoxicity is mediated by the membrane attack complex of C, as cytotoxicity was not observed with C5-depleted serum and could be restored by addition of purified C5. Thus, to the extent that the *in vitro* model we are using is relevant to *in vivo* hyperacute rejection, in swine to primate combinations hyperacute rejection is initiated when natural antibodies bind to porcine endothelium and trigger classical pathway activation. In this combination we found no evidence of antibody-independent activation of either the classical or alternative pathway by the endothelium of the donor organ. This observation was confirmed in studies of heart xenografts in rhesus monkeys with normal serum C levels but in which natural antibodies had been depleted (10). In two cases the grafts continued to function without evidence of rejection when the animals were sacrificed at days 1 and 8 post-transplant, respectively. In repeated samples serum C activity was normal. Very little or no C was deposited in the surviving xenografts, suggesting that in monkeys with normal C levels, C was not activated directly by a porcine xenograft and would not by itself trigger xenograft rejection (10).

ENDOTHELIAL CELL ACTIVATION BY ANTIBODY AND COMPLEMENT: RELEASE OF HEPARAN SULFATE PROTEOGLYCAN

Intravascular coagulation is a prominent feature of hyperacute rejection. In normal blood vessels, coagulation at endothelial cell surfaces is inhibited by antithrombin III which is activated by endothelial cell-associated heparan sulfate proteoglycan. To test whether natural antibody and C might mediate a change in heparan sulfate proteoglycan, the proteoglycans of cultured porcine endothelial cells were labelled with [^{35}S]-sulfate and studied after exposure to natural antibody and C in human serum. This treatment releases 5% of labeled molecules at 4 min and greater than 50% at 60 min, preceding irreversible cell injury. Release of heparan sulfate depended upon natural antibody binding to the cells and classical C pathway activation as it was not observed with sera that were depleted of natural antibodies or of C2, or with heat-inactivated serum. Released proteoglycans consisted of heparan sulfate chains or heparan sulfate glycopeptides (8).

When different normal human sera were added to ^{35}S-labelled endothelial cells, 4% to 65% of heparan sulfate was released after 60 min of incubation. Heparan sulfate release correlated with serum levels of IgM antibodies that bound to porcine endothelial cells and also with deposition of iC3b on the cells, but not with serum C activity (11). The loss of heparan sulfate was found to be triggered by C5a and natural antibody acting in combination on endothelial cells (12).

Heparan sulfate is a biologically active, anionic saccharide which in normal blood vessels promotes the integrity of endothelium, inhibits thrombus formation, tethers superoxide dismutase to the cell, and attaches the endothelium to the underlying extracellular matrix. Thus, C-mediated loss of heparan sulfate accompanying endothelial cell activation may contribute to the pathogenesis of tissue damage in hyperacute rejection.

A NOVEL APPROACH TO PREVENT XENOGRAFT HYPERACUTE REJECTION USING HOST SPECIES MEMBRANE-ASSOCIATED COMPLEMENT INHIBITORS

Inhibition of C activation in a clinically useful manner has not yet been accomplished. Although cobra venom factor inactivates C effectively (3,4), its administration to humans has not been possible, because of formation of neutralizing antibodies upon repeated administration and the risk of complications caused by C-derived fragments. Therefore, we have proposed an alternative approach to C inhibition that takes advantage of the presence in endothelial cells of several membrane-associated regulators of C activation. These proteins are inhibitory to homologous C and may have little inhibitory capacity upon C from a different species(15,16); thus, it is possible that porcine C inhibitors present on porcine endothelial cells might not inhibit human C efficiently. We hypothesized that incorporation of human membrane C inhibitors into the vascular endothelial cell membranes of porcine organs will cause marked inhibition of human C (2,13). We tested this hypothesis with purified human DAF added to porcine endothelial cells to

ascertain whether it would incorporate into the endothelial cells and protect them from the cytotoxic effect of human C. DAF was selected because it acts relatively early in the course of the C reaction, at the level of the C3 and C5 convertases of both classical and alternative pathways. Additionally, by virtue of its phophatidylinositol moiety, DAF can be inserted extrinsically into cell membranes (15).

Human DAF was purified from red cell membranes and labelled with ^{125}I to assay its incorporation into porcine endothelial cells as a function of the concentration of DAF in the added medium. We found a linear dose-response relationship between DAF activity added to the medium and number of DAF molecules incorporated per endothelial cell. The maximum dose of 2,000 units of DAF per well resulted in the incorporation of 2.5×10^5 molecules of DAF per endothelial cell (0.20-0.35% of the added DAF).

To investigate the functional activity of purified human DAF incorporated into porcine endothelial cells, different amounts of DAF were added to monolayers of endothelial cells, which were incubated at 37°C for various periods of time. After washing, the endothelial cells were treated with human serum as source of natural antibody and C. Results (Fig. 1) show marked reduction in C-mediated cytotoxicity of the treated endothelial cells, indicating that human DAF incorporated into porcine endothelial cell membranes and functioned efficiently (13). The inhibition of C-mediated cytotoxicity appeared to be related to the amount of DAF added to the cells and the time of incubation at 37°C. Additional dose-response studies showed a reduction in C-mediated cytotoxicity of endothelial cells proportional to the dose of DAF used to treat the cells. These experiments demonstrate that human DAF readily incorporates into porcine endothelial cells *in vitro*, where it functions as a very effective C inhibitor.

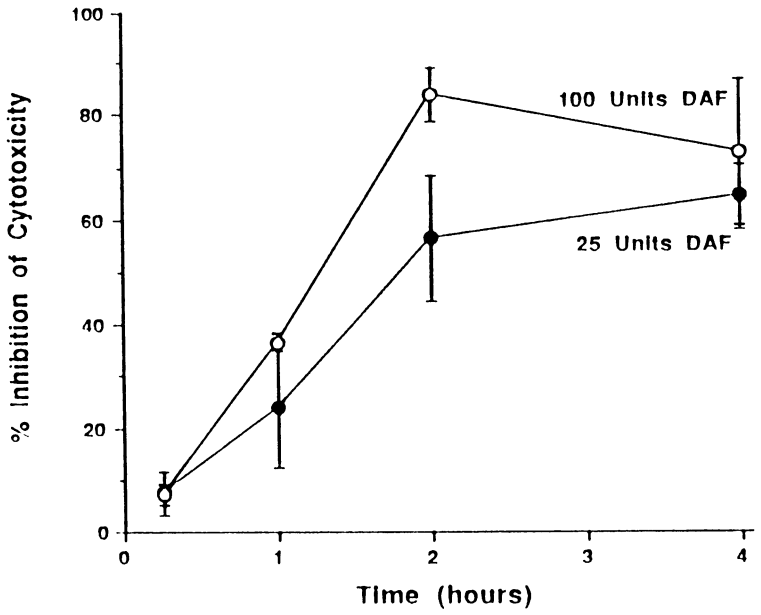

Figure 1. The inhibitory activity of human DAF on cytotoxicity of porcine endothelial cells induced by human C. Purified DAF was incubated with endothelial cells at 37°C for the indicated time periods. DAF inhibitory activity was tested using human serum as source of C and natural antibodies to porcine endothelial cells. Level of cytotoxicity in the absence of DAF was 19%. Results represent mean and range of duplicate wells. (From reference 13, with permission).

CONCLUDING REMARKS

It has been generally held that hyperacute rejection of xenografts is initiated by C activation triggered by natural antibodies against the vascular endothelium of the graft or possibly by the C activating capacity of the foreign endothelium itself (1). Our findings described herein and reviewed elsewhere (2) support that view. In pig to primate combinations, hyperacute rejection is initiated when natural antibodies

bind to porcine endothelium and trigger activation of the classical pathway. In this combination no evidence was obtained of antibody-independent direct activation of either pathway by the endothelim of the donor organ. It has been suggested that in guinea pig to rat (17) and in rabbit to newborn pig (18) xenografts the alternative C pathway may initiate rejection because recipients have been found to have little or no detectable anti-donor antibodies. However, other investigators have suggested that hyperacute rejection in the guinea pig to rat model may be due to low levels of IgM natural antibodies (19). C-derived products activate the vascular endothelium, platelets and neutrophils, vasoactive substances are produced and coagulation is activated. These events lead to hyperacute rejection, which obliterates the xenograft blood circulation within minutes to a few hours after initiation of perfusion of the grafted organ with host blood.

Identification of the steps of the C reaction that participate in hyperacute rejection is necessary to define the C-mediated mechanism responsible for massive rejection of a discordant xenograft. Various C fragments may participate in production of tissue pathology in hyperacute rejection. We found that C5a in conjunction with natural antibodies causes the cleavage and loss of heparan sulfate from the endothelium (12). The loss of heparan sulfate might contribute to disruption of blood vessel integrity and also to intravascular coagulation, which are components of hyperacute rejection. C3bi bound to endothelium markedly enhances leukocyte adherence (20,21). The membrane attack complex, in sublytic amounts, can produce loss of membrane fragments and vesiculation, which in turn promote coagulation (22). These processes may initiate thrombosis and necrosis of the grafted organ before C-mediated endothelial cell killing takes place (2). In C6-deficient rabbits transplanted with dog (6) or cat (7) hearts there was prolongation of xenograft survival. In C6-deficient rabbits the C-mediated activities that are due to activation of C3 remain intact but generation of C5a may be defective (7). These studies do not exclude an important role of C3a, C3b, C5a, or other factors in hyperacute rejection but suggest an important role for the membrane attack complex.

Precise understanding of these C-mediated mechanisms may be useful in designing therapeutic intverventions to avert hyperacute xenograft rejection. A novel approach that we have proposed (13) is based on current knowledge about the role of membrane-associated inhibitors of the C reaction (15,16). When expressed on the vascular endothelium of a xenogeneic donor organ, membrane C inhibitors of the host species could interfere with the contribution of C to xenograft hyperacute rejection. This approach could potentially be used in xenotransplantation of porcine organs to humans, together with other manipulations such as removal of host anti-porcine endothelial cell antibodies, to help abrogate hyperacute rejection.

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